In Vitro Ruminal Degradation and Synthesis of Protein on Fractions Extracted from Alfalfa Hay and Silage[1]

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ABSTRACT

Net release of degraded N as NH_3 and total AA plus microbial protein synthesis, quantified from incorporation of $^{15}NH_3$ into microbial protein, was used to estimate the rate and extent of in vitro degradation of protein fractions isolated from alfalfa hay and silage. Seven proteins (casein, alfalfa hay, alfalfa silage, extracts from alfalfa hay and silage, and residues from alfalfa hay and silage) were studied. Results from $(NH_4)_2SO_4$ and SDS-PAGE fractionations suggested that soluble proteins in alfalfa hay and silage differed in susceptibility to proteolytic attack. Although the net release of NH_3 plus total AA N from alfalfa silage and alfalfa silage extract was twofold greater than that from alfalfa hay and alfalfa hay extract, net microbial protein synthesis on alfalfa hay and alfalfa hay extract was 33 and 43% greater. Despite greater NPN content in alfalfa silage, protein degradation rate and estimated escape were similar for intact alfalfa hay (0.103/h and 43%) and silage (0.067/h and 43%). This result might be explained by the less efficient microbial utilization of silage NPN, greater protozoal numbers on hay, greater soluble true protein in hay, or differences in molecular mass and stability of soluble proteins in hay versus silage. Use of a two-compartment model, based on water-soluble and insoluble CP fractions assumed to pass with the liquid and solid phases, respectively, yielded RUP estimates for alfalfa hay and silage that were similar to NRC estimates.

(**Key words**: alfalfa silage, alfalfa hay, microbial protein, protein degradation and yield)

Abbreviation key: **AH** = alfalfa hay, **AHE** = AH extract, **AHR** = AH residue, **AS** = alfalfa silage, **ASE** = AS extract, **ASR** = AS residue.

INTRODUCTION

Alfalfa forage, conserved as silage or hay, is a major dietary component for lactating cows. However, alfalfa protein, particularly that in alfalfa silage (**AS**), is poorly utilized because of extensive degradation in the rumen (23). The NRC (26) reported that the RUP content of alfalfa hay (**AH**) was 18% greater than that of AS. The NPN content of AS typically ranges from about 50% (9) to as high as 87% (25) of total N. Diets based on alfalfa were more limiting in resistant protein than were diets based on corn silage supplemented with soybean meal (5). Cows fed diets containing a large proportion of AS during early lactation had reduced milk yield and milk protein content (34). Postruminal infusion of protein (as casein) in lactating cows fed a diet containing 98% of DM from AS increased milk and protein yields by 5.5 and 0.18 kg/d, respectively, and infusion of energy (as glucose) did not increase yield (12). These results indicated that protein, not energy, was the first-limiting nutrient for milk yield in cows fed diets high in AS. For lactating dairy cows fed AH or AS as the sole forage, addition of 3% fish meal, a high source of RUP, increased milk protein yield by 30 and 100 g/d, respectively (7). Poor performance on diets high in AS might result from inadequate capture of dietary N as absorbable protein.

Soluble proteins differ markedly in ruminal degradability (28). Solubility and structural properties such as disulfide bridges, surface charge, hydrophobicity, and folding may be involved in determining protein susceptibility to microbial proteolytic attack (28, 29). Thus, the value of protein solubility as an indicator of protein degradability is limited. Moreover, CP solubility does not distinguish between N in soluble true protein and NPN. Electrophoretic techniques have been used to monitor the effects of drying, wilting, and ensiling on soluble forage proteins (13, 16, 20).

Recently, a new in vitro procedure was developed to estimate the rate and extent of ruminal protein degradation using $^{15}NH_3$ to quantify microbial incorporation of protein degradation products (17). Our objectives were to use this procedure 1) to estimate

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rate and extent of degradation of proteins in intact AS and AH, in the water-soluble extracts from AS (**ASE**) and AH (**AHE**), and in the water-insoluble residues from AS (**ASR**) and AH (**AHR**); 2) to measure microbial protein synthesis on proteins in AS and AH, in soluble ASE and AHE, and in insoluble ASR and AHR; and 3) to estimate protein degradation and escape of AS and AH with a two-compartment model using the proportions and degradation rates of soluble and insoluble proteins plus literature values for passage rates of liquid (soluble) and solid (insoluble) phases.

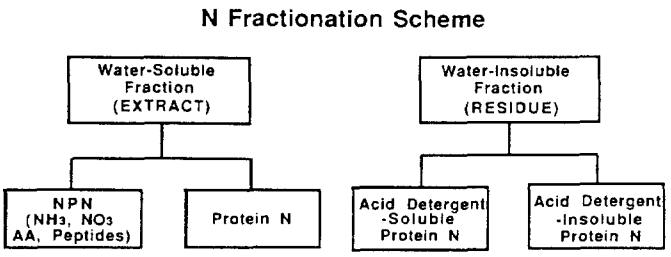

Figure 1. Schematic representation of N fractionation used on alfalfa hay and silage.

MATERIALS AND METHODS

Sample Description and Analysis

Four composite samples of freeze-dried AS and four of air-dried AH, two each from two feeding studies (7), were ground through a 1-mm screen (Wiley mill; Arthur H. Thomas, Philadelphia, PA) and analyzed for total N by the Kjeldahl procedure (1) using a copper digestion catalyst (Kjeltabs®; Tecator Inc., Herndon, VA) and for NDF (32), ADF (32), and ADIN (15). Triplicate samples containing 38.5 mg of N each were mixed with 35 ml of distilled water in centrifuge tubes. After capping, tubes were placed in a 39°C water bath equipped with a reciprocal shaker. Tubes were warmed without shaking for 30 min; after shaking for 60 min, samples were filtered through Whatman number 1 filter paper (Arthur H. Thomas), and 10-ml aliquots were analyzed for N by the Kjeldahl procedure (1). Soluble N was expressed as a percentage of the total N in the sample originally added to centrifuge tubes. Soluble true protein was assayed by the dye-binding procedure of Bradford (3) using a Bio-Rad kit (Bio-Rad Laboratories, Hercules, CA).

Extraction Procedure

The AS and AH were extracted with distilled water to prepare soluble and insoluble CP fractions (Figure 1) for determination of rate and extent of ruminal protein degradation. Extractions were made with distilled water, rather than buffer, to avoid the presence of excess buffer salts in the freeze-dried extracts. Ground samples of AS or AH (30 g of DM) were mixed with distilled water (600 ml) in 1-L capped bottles with continuous stirring for 1 h at 39°C. The mixture was centrifuged at 31,000 × *g* for 15 min (4°C) and then filtered through Whatman number 1 filter paper. The water-soluble extracts (ASE and AHE) and the insoluble residues (ASR and AHR) were freeze-dried and analyzed for total N content by Kjeldahl analysis (1).

$(NH_4)_2SO_4$ Fractionation and SDS-PAGE of Soluble Proteins

Samples of ASE and AHE were mixed with 10% (wt/vol) TCA and centrifuged at 15,000 × *g* at 4°C for 5 min. Pellets were washed twice with ethanol:ether (1:1, vol/vol) and resuspended in homogenization buffer [50 m*M* sodium phosphate (pH 7.2), 1 m*M* phenyl methyl sulfonyl fluoride, and 10 m*M* EDTA]. Then, samples were fractionated sequentially by continuous stirring for 2 h at 4°C with 20, 50, and then 100% (wt/vol) $(NH_4)_2SO_4$, followed by centrifugation at 20,000 × *g* for 10 min at 4°C. Sedimented proteins in the pellets were resuspended in, and dialyzed against, 20 m*M* sodium phosphate buffer (pH 7.2). Samples were recentrifuged (20,000 × *g* for 10 min at 4°C) to remove sediment, and true protein was determined in the supernatants (3). Equal amounts of total protein (40 μg) from the three $(NH_4)_2SO_4$ fractions of AHE and ASE were resolved using 12.5% SDS-PAGE (19).

In Vitro Procedure

About 2 h after the morning feeding, equal amounts of digesta were collected from two ruminally cannulated, lactating cows fed a TMR containing AS, corn silage, cracked shell corn, and soybean meal (17). Digesta were mixed and processed to prepare the ruminal inoculum, as was described earlier (11); pH and NH_3 concentration (8) were determined. Seven protein sources [casein (Sigma Chemical Co., St. Louis, MO), AH, AS, AHE, ASE, AHR, and ASR] were incubated at 39°C for 6 h in spinner flasks (Bellco, Vineland, NJ) with continuous stirring using the ruminal in vitro procedure of Hristov and Broderick (17), except that 6.74 mg of $(NH_4)_2SO_4$ enriched with 73 atom % of excess ^{15}N were added to each

flask; duplicate flasks for blanks and for each protein source were used in each incubation; four separate incubations were conducted. Each flask contained 360 ml of final incubation mixture. Protein sources were added to the flasks to provide 0.125 mg of N/ml of incubation mixture or 45 mg of N/flask. Because N contents varied, the equivalent amount of DM added to each flask ranged from 300 (ASE) to 1770 mg (ASR). Protozoa numbers were determined microscopically (30), and pH was recorded in each flask at the end of each incubation.

Duplicate samples were taken from each flask by increasing the stirring speed of the flask and aspirating the slurry using a 25-ml serological pipet with a large tip opening (no. 7087; Corning Co., Corning, NY). Methods described earlier (17) were used to analyze for NH_3 and total AA (8), for non-NH_3 N, and for ^{15}N enrichment in NH_3, isolated bacterial non-NH_3 N, and total solids non-NH_3 N by isotope ratio mass spectrometry (Finnegan MAT mass spectrometer; San Jose, CA). Percentage of protein degradation (PPD) of each protein was computed from net release of protein N (substrate flask minus blank flask) as described (17), except that total AA N was included with NH_3 N (in TCA soluble NPN) plus microbial N as degraded protein N:

$$PPD = \left[\frac{(\text{net } NH_3 \text{ N} + \text{TAA N})_6 - (\text{net } NH_3 \text{ N} + \text{TAA N})_0 + \text{net } MN_6}{N_p - (\text{net } NH_3 \text{ N} + \text{TAA N})_0 - ADIN_p}\right] \times 100 \quad [1]$$

where TAA N is total AA N; Net $(NH_3$ N + TAA N$)_0$ and Net $(NH_3$ N + TAA N$)_6$ are the blank corrected NH_3 N plus total AA N (milligrams per deciliter) present at 0 and 6 h, respectively; Net MN_6 is the blank corrected microbial non-NH_3 N (milligrams per deciliter) synthesized after 6 h; and N_p and $ADIN_p$ are, respectively, the protein N and ADIN (milligrams per deciliter) added at 0 h with the protein. Fraction B (degradable intact protein), fractional degradation rate (k_d), and percentage of estimated ruminal escape (with and without ADIN) were computed as follows:

$$\text{Fraction B, percentage of total N} = [N_p - (\text{net } NH_3 \text{ N} + \text{TAA N})_0 - ADIN_p] \times 100, \quad [2]$$

$$k_d/h = \left[-\ln \frac{(100 - PPD)}{100}\right]/6 \text{ h}, \quad [3]$$

$$\text{Estimated escape} = \left[\frac{k_p}{(k_d + k_p)} \times B\right] + ADIN_p, \quad [4]$$

and

$$\text{Estimated escape (corrected for ADIN)} = \left[\frac{k_p}{(k_d + k_p)}\right] \times B \quad [5]$$

where k_p is the fractional rate of passage from the rumen, and net $(NH_3$ N + TAA N$)_0$, N_p, $ADIN_p$, and PPD are as defined previously. Fractional rates of passage assumed (6) for these computations were k_p = 0.06/h for intact (casein, AS, and AH) and insoluble proteins (ASR and AHR), and k_p = 0.12/h for soluble proteins (ASE and AHE).

Four separate incubations were carried out: 1) two runs each with 20 flasks with duplicate sets of 10 (blank and casein, plus 4 composites of AS and 4 of AH); and 2) two runs each with 28 flasks with duplicate sets of 14 [blank and casein, plus 2 each of AS, ASE, ASR, AH, AHE, and AHR—one set for each feeding trial (7) in each run]. Degradability data were computed within each set (1 blank and 9 substrate flasks for the first two runs and 1 blank and 13 substrate flasks for the second two runs) from net differences between the blank and substrate flasks. During the four runs, 24 separate observations of each variable were made for AS and AH, plus 8 for blanks and casein; 8 observations of each variable were made for ASE, ASR, AHE, and AHR during the last two runs.

Statistical Analysis

Statistical analysis was conducted with the general linear models procedures of SAS (33) using the type III sum of squares. The model included treatment (n = 8 for blank, casein, ASE, ASR, AHE, and AHR flasks; n = 24 for AS and AH flasks), incubation (n = 4), replicate (n = 2), and feeding trial (7) (n = 2) plus interactions of treatment × incubation, replicate × trial, and replicate × incubation. The replicate × incubation interaction was used as the error term in the analysis to detect differences because of treatment; when effects ($P < 0.05$) were detected, mean separation was by least significant difference at P = 0.05.

RESULTS AND DISCUSSION

Forages (Table 1) were typical in composition of high quality AH and AS (26). As observed earlier (7), NDF and ADF were not different between AH and AS, but CP content of AS was about two percen-

TABLE 1. Composition of alfalfa hay (AH) and alfalfa silage (AS).

Item	AH	AS	SE	$P > F^1$
DM, %	85.32	40.53	1.41	<0.001
NDF, % of DM	38.34	37.74	3.47	0.738
ADF, % of DM	28.61	29.03	3.41	0.813
Ash, % of DM	9.64	10.40	0.36	0.001
CP, % of DM	18.19	20.12	1.22	0.019
ADIN, % of total N	9.96	4.97	0.43	<0.001
Water-soluble N, % of total N	24.83	42.78	0.82	<0.001
Soluble protein N, % of total N	2.10	0.50	0.07	<0.001
NPN,[2] % of total N	22.73	42.28	0.06	<0.001

[1]Probability of an effect of forage source.

[2]NPN = Water-soluble N − soluble protein N.

tage units greater than that of AH. The ADIN content of AH was twice that of AS. The NPN contents (Table 1; Figure 1) were lower (AS) and higher (AH) than values reported previously (7); however, those samples were from another set that was assayed using a different solvent. Water-soluble true protein was 1.2 and 8.4% of total soluble N for AS and AH, respectively (Table 1). Composition differences in the N fractions in AH and AS might be explained by the method of conservation. The sevenfold greater soluble true protein in AH likely resulted from the extensive conversion of soluble proteins in AS to NPN, which was a result of the well-known action of plant and microbial proteolysis during ensiling (20, 25). Drying during hay making probably also contributed to differences among individual proteins. Messman et al. (24) observed that drying fresh alfalfa to AH reduced the amount of total soluble protein that was identifiable electrophoretically by about 25%, and ensiling reduced it by more than 90%. Fraction 1 protein, the principal soluble protein in green forages (21), was only partly lost during wilting but was completely degraded after 2 d of ensiling (13).

Water-soluble proteins in AHE and ASE were fractionated using 20, 50, and 100% (wt/vol) $(NH_4)_2SO_4$; equal amounts of true protein (40 μg) from each $(NH_4)_2SO_4$ fraction of AHE and ASE were separated using 12.5% SDS-PAGE. All $(NH_4)_2SO_4$ fractions showed differences in major proteins (Figure 2). For example, the 20-kDa protein that was in all AHE fractions was absent from ASE fractions, indicating that this protein was completely degraded during ensiling. Another protein of 24-kDa molecular mass, present in the 50% (wt/vol) $(NH_4)_2SO_4$ fractions from ASE, was absent from AHE extracts. This protein might have resulted from partial hydrolysis of an alfalfa protein during ensiling or might have been a microbial protein formed during fermentation in the silo. Other proteins, such as the dominant 40-kDa protein observed in the 50% (wt/vol) $(NH_4)_2SO_4$ fractions, were present in both ASE and AHE; this protein was quite stable, regardless of method of preservation. Thus, soluble proteins from AS and AH differed in stability, probably because of differences in susceptibility to proteolytic attack. The SDS-PAGE technique might be useful for monitoring the effect of treatments designed to increase the stability of major alfalfa proteins to the degradative action in the silo and in the rumen.

Characteristics of the in vitro incubations at 6 h are reported in Table 2. The addition of large amounts of fermentable carbohydrates was intended to equalize fermentable energy in all incubations. However, lower pH in the incubations with AH, AS, AHE, and ASR might reflect greater VFA production

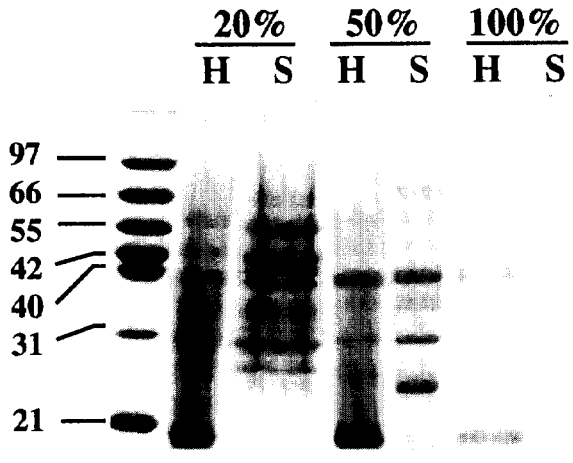

Figure 2. Electrophoretic analysis, using 12.5% SDS-PAGE, of water-soluble extracts from alfalfa hay (H) and alfalfa silage (S). Concentrations of $(NH_4)_2SO_4$ used in fractionation (20, 50, and 100%, wt/vol) are shown along the top. Total true protein applied was 40 μg per lane (3); gels were stained with Coomassie blue. Migration of the molecular mass markers is shown on the left.

TABLE 2. Data after 6-h in vitro incubations with blanks, intact proteins, water-soluble extracts, and water-insoluble residues from alfalfa hay and silage.[1]

Source	Blank (n = 8)	Casein (n = 8)	Intact forage (n = 24) AH	Intact forage (n = 24) AS	Soluble N (n = 8) AHE	Soluble N (n = 8) ASE	Insoluble N (n = 8) AHR	Insoluble N (n = 8) ASR	SE	$P > F^2$
pH	6.41[a]	6.45[a]	6.26[c]	6.24[c]	6.25[c]	6.40[a]	6.40[a]	6.33[b]	0.02	0.037
Protozoa, number/ml × 10^5	1.76[c]	1.13[d]	2.73[b]	1.69[c]	0.97[d]	0.99[d]	3.58[a]	2.00[c]	0.12	0.002
NH_3 N, mg/dl	13.07[d]	19.62[a]	15.70[c]	16.13[c]	15.39[c]	18.10[b]	11.38[e]	9.03[f]	0.32	<0.001
TAA N, mg/dl	1.07[cd]	1.40[cd]	1.27[cd]	2.13[b]	1.51[c]	3.28[a]	1.00[d]	0.98[d]	0.12	0.048
TS N, mg/dl	32.01[f]	35.32[e]	43.30[b]	41.96[c]	38.30[d]	35.92[e]	42.32[c]	45.81[a]	0.29	<0.001
NH_3 ^{15}N, atom % excess	1.037[a]	0.862[e]	0.940[c]	0.899[d]	0.792[f]	0.772[f]	0.974[b]	0.925[cd]	0.010	0.012
TS ^{15}N, atom % excess	0.442[a]	0.301[e]	0.378[d]	0.400[b]	0.385[cd]	0.390[bcd]	0.393[bc]	0.437[a]	0.005	0.034
Bacterial solids ^{15}N, atom % excess	0.578[b]	0.333[f]	0.508[d]	0.545[c]	0.461[e]	0.474[e]	0.586[b]	0.664[a]	0.007	<0.001
MN, mg/dl	24.09[d]	32.03[a]	32.33[a]	30.57[b]	30.65[b]	27.73[c]	27.94[c]	29.70[b]	0.36	0.046

[a,b,c,d,e,f]Means in the same row without a common superscript differ ($P < 0.05$).

[1]AH = Alfalfa hay, AHE = AH extract, AHR = AH residue, AS = alfalfa silage, ASE = AS extract, ASR = AS residue, MN = microbial N, and TAA N = total AA N.

[2]Probability of a treatment effect.

from fermentation of the extra carbohydrates added with those four substrates. Protozoal numbers also varied among flasks. Generally, protozoal numbers were greater in incubations containing the intact forages (AH and AS) and residues (AHR and ASR) than in the other incubations (Table 2). Means of data that were used to compute net extent of protein degradation also are presented in Table 2. Enrichment of NH_3 with ^{15}N was negatively related to NH_3 concentration ($r = -0.58$); NH_3 concentration probably accounted for only part of the variation ($r^2 = 0.33$) in NH_3 enrichment because NH_3 was simultaneously produced from AA deamination and was utilized for microbial growth.

Data used to compute the rate of ruminal protein degradation and to estimate ruminal protein escape

TABLE 3. Computation of in vitro degradation of protein in casein, intact alfalfa hay and silage, water-soluble extracts, and water-insoluble residues from alfalfa hay and silage.[1]

Source	Casein (n = 8)	Intact forage (n = 24) AH	Intact forage (n = 24) AS	Soluble N (n = 8) AHE	Soluble N (n = 8) ASE	Insoluble N (n = 8) AHR	Insoluble N (n = 8) ASR	SE	$P > F^2$
ADIN, mg/dl	...	1.25	0.62	...	...	1.65	1.09	...	...
Net NH_3 N + TAA N (0 h), mg/dl	−0.01[d]	1.63[c]	2.55[b]	2.60[b]	4.74[a]	0.19[d]	0.61[d]	0.19	0.046
Net NH_3 N, mg/dl	6.06[a]	0.81[d]	1.17[d]	2.76[c]	5.47[b]	−1.29[e]	−3.63[f]	0.184	<0.001
Net TAA N, mg/dl	0.15[cd]	0.24[cd]	0.98[b]	0.54[c]	2.27[a]	0.05[d]	0.01[d]	0.14	0.016
Net NH_3 N + TAA N (6 h), mg/dl	6.20[b]	0.97[e]	1.82[d]	3.30[c]	7.74[a]	−1.22[f]	−3.60[g]	0.18	<0.001
Net microbial N, mg/dl	5.89[cd]	7.20[b]	5.43[d]	9.71[a]	6.78[bc]	7.00[bc]	8.76[a]	0.33	0.043
Total degraded N, mg/dl	12.09[a]	5.54[c]	4.08[d]	10.40[b]	9.78[b]	3.94[d]	3.45[d]	0.30	0.042
Protein degradation, %	96.7[a]	44.3[c]	32.7[d]	83.2[b]	78.2[b]	31.5[d]	27.6[d]	2.4	0.042
Fractional degradation rate, /h	0.486[a]	0.103[c]	0.067[cd]	0.273[b]	0.285[b]	0.064[d]	0.054[d]	0.014	0.027
Escape, %	11.4[e]	43.4[b]	42.6[b]	25.1[c]	19.8[d]	55.1[a]	54.3[a]	1.2	0.048
Escape − ADIN, %	11.4	33.4	37.6	25.1	19.8	41.9	45.6	1.2	0.113

[a,b,c,d,e,f,g]Means in the same row without a common superscript differ ($P < 0.05$).

[1]AH = Alfalfa hay, AHE = AH extract, AHR = AH residue, AS = Alfalfa silage, ASE = AS extract, ASR = AS residue, and TAA N = total AA N.

[2]Probability of a treatment effect.

are presented in Table 3. Net release of NH_3 N from AHE and ASE was greater than that from AH and AS. Net release of NH_3 N was negative from both ASR and AHR, indicating net uptake of NH_3 N from the medium for microbial growth; net uptake of NH_3 N was greater in ASR than in AHR incubations (Table 3). Net accumulations of total AA N were small, except for the ASE incubations; total AA N accounted for about 23% of degraded protein for ASE. Although net release of N as NH_3 N plus total AA from AS and ASE was about twofold greater than that from AH and AHE, net microbial N synthesis on AH and AHE was, respectively, 33 and 43% greater ($P < 0.001$) than on AS and ASE (Table 3). This result indicated that, in our in vitro system, microbial utilization of the degraded N fraction from silage, which was largely NPN, was less efficient than utilization of degraded N from hay. In incubations with insoluble residues, in which little or no NPN or soluble protein was added to the inoculum, net microbial protein synthesis was greater on ASR than on AHR (Table 3). Hristov and Broderick (18) found that, compared with AH, less degraded CP from AS was recovered as net microbial protein synthesis in the rumen of lactating cows. Greater net formation of microbial protein on AH might be attributed to preferential utilization of AA and peptides by mixed ruminal microorganisms when NH_3 is in excess (31, 36). Lower ^{15}N enrichment of bacterial solids N in AH than in AS incubations (Table 2) probably reflected relatively lower incorporation of the $^{15}NH_3$ tracer because of relatively greater uptake of AA and peptides. Chen et al. (10) also showed that individual species of ruminal bacteria grew faster with peptides present in the medium. Greater utilization by ruminal microbes of degraded N from intact proteins than from NPN sources also might result from improved synchrony between rates of N release and energy fermentation (27).

As expected, casein had the most rapid degradation rate and the lowest estimated ruminal escape of the proteins studied (Table 3). Generally, soluble proteins from the alfalfa forages were more susceptible to ruminal degradation than were insoluble proteins (28, 29). However, there were no significant differences between hay and silage in degradation rate and estimated ruminal protein escape for intact forages or for corresponding insoluble residues; only AHE had greater estimated escape than did ASE (Table 3). With the intact forages, degradation rate and estimated escape (when discounted for ADIN, which may be considered totally unavailable) indicated that protein in AS was numerically less degradable than that in AH, despite the higher NPN content of AS (Table 1). The more rapid degradation rate for casein than for the water-soluble proteins extracted from hay and silage (AHE and ASE) suggested that ruminal protein degradability was not related to solubility only. Nugent et al. (28), in studies with four soluble proteins (casein, fraction I leaf protein, bovine serum albumin, and bovine submaxillary mucoprotein), concluded that rate of ruminal proteolysis was not dependent on solubility, but was related to the structural folding of the protein molecule. This relationship might explain the very high degradation rates we found for casein. Casein is not typical of common feed proteins because it lacks disulfide bridges and has a high content of phosphoserine residues, which might increase its affinity to proteolytic attack (29).

Protein degradation might be influenced partly by protozoa. Protozoal numbers were greater in AH than in AS incubations (Table 2), and degradation rate for AH was numerically greater than for AS (Table 3). Protozoa were shown to have only 10% of the proteolytic activity of bacteria in vitro (14); however, they may contribute a proportionately greater amount to ruminal protein degradation. Bacteria were reported to act principally in degradation of soluble proteins (4, 29), and protozoa might act through engulfment of feed particles (and bacteria) and in degradation of insoluble proteins (35). Compared with the soluble fractions (ASE and AHE), protozoal numbers were greater (Table 2) in incubations with intact proteins (AH and AS) and with insoluble residues (AHR and ASR); protozoal numbers were greatest with AHR (Table 2). Feed particles of larger size might favor greater numbers of protozoa (2). Increased protozoal numbers were associated with decreased net production of NPN (net NH_3 plus total AA N) in the AHR and ASR incubations (Table 3). Chloroplasts released from plant material were engulfed quantitatively by entodiniomorphid protozoa, but without the release of soluble N compounds to the medium (22). Thus, differences in chemical nature and anatomical organization of the plant material might influence protozoal numbers and, hence, protein degradation (35) as well as the physical and chemical properties of proteins mentioned earlier.

Total degraded N after 6 h was similar for ASE, AHE, and casein (Table 3); degradation rates for AHE (k_d = 0.273/h) and ASE (k_d = 0.285/h) were similar. Although degradation rates of these fractions were rapid, intact protein in AHE and ASE represented only 2.1 and 0.5% (Table 1) of the total N in AH and AS, respectively. Degradation rate for AHR (0.064/h) was similar to that for ASR (0.054/h)

(Table 3). Degradation rates for AH and AS were 0.103 and 0.067/h, respectively.

A two-compartment model (6) was used to compute estimated ruminal degraded protein (EDP) and estimated ruminal escaped protein (EEP), as a percentage of total N, for AS and AH:

$$EDP = A + \left[B_s \times \frac{k_{ds}}{(k_{ds} + k_{ps})}\right] + \left[B_i \times \frac{k_{di}}{(k_{di} + k_{pi})}\right]$$

and

$$EEP = \left[B_s \times \frac{k_{ps}}{(k_{ds} + k_{ps})}\right] + \left[B_i \times \frac{k_{pi}}{(k_{di} + k_{pi})}\right] + C = 100 - EDP$$

where A is the percentage of total CP as NPN; B_s and B_i are the percentages of total CP as soluble and insoluble proteins; C is the percentage of total CP as ADIN; k_{ds} and k_{di} are degradation rates, estimated in vitro, for the soluble and insoluble proteins, respectively; and k_{ps} and k_{pi} are the ruminal passage rates for soluble and insoluble phases, set equal to 0.12 and 0.06/h, respectively. For the purposes of these calculations, water-soluble NPN (Table 1), rather than net NH_3 plus total AA at 0 h (Table 3), was used as the measure of fraction A. These equations were used to compute EDP and EEP for AH and AS:

$$EDP\ (AH) = 22.73 + \left[2.10 \times \frac{0.273}{(0.273 + 0.12)}\right] + \left[65.21 \times \frac{0.103}{(0.103 + 0.06)}\right]$$

$$= 22.73 + 1.46 + 41.21 = 65.4\% \text{ of total CP;}$$

$$EDP\ (AS) = 42.28 + \left[0.50 \times \frac{0.285}{(0.285 + 0.12)}\right] + \left[52.25 \times \frac{0.067}{(0.067 + 0.06)}\right]$$

$$= 42.28 + 0.35 + 27.56 = 70.2\% \text{ of total CP;}$$

and

$$EEP\ (AH) = 100 - 65.4 = 34.6\% \text{ of total CP;}$$

$$EEP\ (AS) = 100 - 70.2 = 29.8\% \text{ of total CP.}$$

Estimated protein escape for AH, computed by this approach (34.6%), was 16% greater than that computed for AS (29.8%). The RUP value reported by the NRC (26) for AH (28%) is 22% greater than that reported for AS (23%).

CONCLUSIONS

Net release of degraded N as NH_3 and total AA, plus microbial protein, quantified from $^{15}NH_3$ incorporation into microbial non-NH_3 N, was used to estimate the rate and extent of in vitro degradation of protein in intact AH and AS, in water-soluble extracts (AHE and ASE), and in water-insoluble residues (AHR and ASR) from AH and AS. Although the water-soluble proteins were more degradable, overall rate and extent of protein degradation were similar for the equivalent fractions. Contribution of NPN to in vitro degradation was greater for ASE than for AHE. Microbial protein synthesis on intact AH and AHE was greater than on AS and ASE and might be related to greater microbial uptake of N from AA and peptides. Use of a two-compartment model, based on soluble and insoluble CP fractions assumed to pass with the liquid and solid phases, yielded similar estimates of ruminal protein degradation and escape for AH and AS that were comparable with NRC (26) values. Protozoal numbers were greater in incubations containing AH and AHR than in those containing AS and ASR. Protozoal activity might have contributed to the surprisingly high extent of protein degradation for AH.

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REFERENCES

1 Association of Official Analytical Chemists. 1980. Official Methods of Analysis. 13th ed. AOAC, Washington, DC.
2 Bonhomme, A. 1990. Rumen ciliates: their metabolism and relationships with bacteria and their hosts. Anim. Feed Sci. Technol. 30:203.
3 Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72:248.
4 Brock, F. M., C. W. Forsberg, and J. G. Buchanan-Smith. 1982. Proteolytic activity of rumen micro-organisms and proteinase inhibitors. Appl. Environ. Microbial. 44:561.
5 Broderick, G. A. 1985. Alfalfa silage or hay versus corn silage as the sole forage for lactating dairy cows. J. Dairy Sci. 68:3262.
6 Broderick, G. A. 1994. Quantifying forage protein quality. Page 200 *in* Forage Quality, Evaluation, and Utilization. G. E. Fahey, Jr., D. R. Mertens, M. Collins, and L. E. Moser, ed. Am. Soc. Agron., Madison, WI.

7 Broderick, G. A. 1995. Performance of lactating dairy cows fed either alfalfa silage or alfalfa hay as the sole forage. J. Dairy Sci. 78:320.
8 Broderick, G. A., and J. H. Kang. 1980. Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and in vitro media. J. Dairy Sci. 63:64.
9 Broderick, G. A., D. B. Ricker, and L. S. Driver. 1990. Expeller soybean meal and corn by-products versus solvent soybean meal for lactating dairy cows fed alfalfa silage as sole forage. J. Dairy Sci. 73:453.
10 Chen, G., H. J. Strobel, J. B. Russell, and C. J. Sniffen. 1987. Effect of hydrophobicity on utilization of peptides by ruminal bacteria in vitro. Appl. Environ. Microbiol. 53:2021.
11 Craig, W. M., B. J. Hong, G. A. Broderick, and R. J. Bula. 1984. In vitro inoculum enriched with particle-associated microorganisms for determining rates of fiber digestion and protein degradation. J. Dairy Sci. 67:2902.
12 Dhiman, T. R., C. Cadorniga, and L. D. Satter. 1993. Protein and energy supplementation of high alfalfa silage diets during early lactation. J. Dairy Sci. 76:1945.
13 Fairbairn, R., I. Alli, and R. E. Baker. 1988. Proteolysis associated with the ensiling chopped alfalfa. J. Dairy Sci. 71:152.
14 Forsberg, C. W., L.K.A. Lovelock, L. Krumholz, and J. G. Buchanan-Smith. 1984. Protease activities of rumen protozoa. Appl. Environ. Microbiol. 47:101.
15 Goering, H. K., and P. J. Van Soest. 1970. Forage Fiber Analyses (Apparatus, Reagents, Procedures, and Some Applications). Agric. Handbook No. 379. ARS-USDA, Washington, DC.
16 Grum, D. E., W. L. Shockey, and W. P. Weiss. 1991. Electrophoretic examination of alfalfa silage proteins. J. Dairy Sci. 74:146.
17 Hristov, A., and G. A. Broderick. 1994. In vitro determination of ruminal protein degradability using ^{15}N-ammonia to correct for microbial nitrogen uptake. J. Anim. Sci. 72:1344.
18 Hristov, A. N., and G. A. Broderick. 1994. Ruminal microbial protein synthesis in cows fed alfalfa silage, alfalfa hay or corn silage and fitted with only ruminal cannulae. J. Dairy Sci. 77(Suppl. 1):386.(Abstr.)
19 Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.) 227: 680.
20 Makoni, N. F., J. A. Shelford, S. Nakai, L. J. Fisher, and W. Majak. 1993. Characterization of protein fractions in fresh, wilted, and ensiled alfalfa. J. Dairy Sci. 76:1934.
21 Mangan, J. L. 1982. The characterization of forage protein. Page 1 *in* Forage Protein Conservation and Utilisation. T. W. Griffiths and M. F. Maguire, ed. Comm. Eur. Commun., Dublin, Ireland.
22 Mangan, J. L., and J. West. 1977. Ruminal digestion of chloroplasts and the protection of protein by glutaraldehyde treatment. J. Agric. Sci. (Camb.) 89:3.
23 Merchen, N. R., and L. D. Satter. 1983. Changes in nitrogenous compounds and sites of digestion of alfalfa harvested at different moisture contents. J. Dairy Sci. 66:789.
24 Messman, M. A., W. P. Weiss, and M. E. Koch. 1994. Changes in total and individual proteins during drying, ensiling, and ruminal fermentation of forages. J. Dairy Sci. 77:492.
25 Muck, R. E. 1987. Dry matter level effects on alfalfa silage quality. 1. Nitrogen transformations. Trans. ASAE (Am. Soc. Agric. Eng.) 30:7.
26 National Research Council. 1989. Nutrient Requirements of Dairy Cattle. 6th rev. ed. Natl. Acad. Sci., Washington, DC.
27 Nocek, J. E., and J. B. Russell. 1988. Protein and carbohydrate as an integrated system. Relationship of ruminal availability to microbial contribution and milk production. J. Dairy Sci. 71: 2070.
28 Nugent, J.H.A., W. T. Johns, D. J. Jordan, and J. L. Mangan. 1983. Rates of proteolysis in the rumen of the soluble proteins casein, Fraction I (18S) leaf protein, bovine serum albumin and bovine submaxillary mucoprotein. Br. J. Nutr. 50:357.
29 Nugent, J. H., and J. L. Mangan. 1981. Characteristics of the rumen proteolysis of fraction I (18S) leaf protein from lucerne (*Medicago sativa* L.). Br. J. Nutr. 46:39.
30 Ogimoto, K., and S. Imai. 1981. Page 161 *in* Atlas of Rumen Microbiology. Jpn. Sci. Soc. Press, Tokyo, Japan.
31 Prins, R. A., J. C. Van Hal-Van Gestel, and G.H.M. Counotte. 1979. Degradation of amino acids and peptides by mixed rumen micro-organisms. Z. Tierphysiol. Tierernaehrg. Futtermittelkd. 42:333.
32 Robertson, J. B., and P. J. Van Soest. 1981. Page 123 *in* The Analysis of Dietary Fiber in Foods. W.P.T. James and O. Theander, ed. Marcel Dekker, New York, NY.
33 SAS® User's Guide: Statistics, Version 5 Edition. 1985. SAS Inst., Inc., Cary, NC.
34 Tessmann, N. J., H. D. Radloff, J. Kleinmans, T. R. Dhiman, and L. D. Satter. 1991. Milk production response to dietary forage:grain ratio. J. Dairy Sci. 74:2696.
35 Ushida, K., J. P. Jouany, and D. I. Demeyer. 1991. Effect of presence or absence of rumen ciliate protozoa on the utilization of fibrous and concentrate diets. Page 625 *in* Physiological Aspects of Digestion and Metabolism in Ruminants. T. Tsuda, Y. Sasaki, and R. Kawashima, ed. Acad. Press, London, England.
36 Wallace, R. J., and M. A. Cotta. 1988. Metabolism of nitrogen containing compounds. Page 217 *in* The Rumen Microbial Ecosystem. P. N. Hobson, ed. Elsevier, London, England.